Original Report

Identification of Tula hantavirus in *Pitymys subterraneus* captured in the Cacak region of Serbia-Yugoslavia

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Background: Atypical serum neutralizing antibody responses to prototype strains of Puumala viruses in some patients with hemorrhagic fever with renal syndrome (HFRS) have long suggested the existence of other hantaviruses in the Balkans.

Objective: To determine the presence of arvicolid rodent-borne Puumala-like hantaviruses in Yugoslavia.

Materials and Methods: Using reverse transcript-polymerase chain reaction, Tula virus RNA was amplified from lung tissues of a European pine vole (*Pitymys subterraneus*) captured in 1987, following an outbreak of HFRS in the Cacak region of Serbia-Yugoslavia.

Results: Sequence analysis of the entire coding region of the S segment and a 948-nucleotide region of the G2 glycoprotein-encoding M segment revealed divergence of approximately 14% from Tula virus strains harbored by European common voles (*Microtus arvalis*) captured in Central Russia and the Czech Republic. However, nearly complete identity was found in the corresponding deduced amino acid sequences. Moreover, phylogenetic trees constructed by the maximum parsimony and neighbor-joining methods indicated that this *Pitymys*-borne hantavirus shared a common ancestry with other Tula virus strains.

Conclusions: The data demonstrate that *Pitymys subterraneus* also serves as a rodent reservoir of Tula virus in Serbia-Yugoslavia. To what extent this represents virus spillover from *Microtus arvalis* warrants further investigation.

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Hantaviruses, the etiologic agents of hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS), are transmitted to humans by the respiratory droplet or airborne route rather than by arthropod vectors.[1,2] However, like other members of the family Bunyaviridae, hantaviruses possess a negative-sense, single-stranded RNA genome comprised of three segments, designated large (L), medium (M), and small (S), which encode an RNA-dependent RNA polymerase, envelope glycoproteins (G1, G2) and a nucleocapsid protein, respectively. All hantaviruses are associated with one or a few closely related rodent species: Hantaan virus is harbored by the striped-field mouse (*Apodemus agrarius*); Dobrava–Belgrade virus by the yellow-necked field mouse (*Apodemus flavicollis*); Seoul virus by the Norway rat (*Rattus norvegicus*); Thailand virus by the bandicoot rat (*Bandicota indica*); Puumala virus by the bank vole (*Clethrionomys glareolus*) and the grey-sided vole (*Clethrionomys rufocanus*); and Prospect Hill virus by the meadow vole (*Microtus pennsylvanicus*).

Recently, a group of disease-causing sigmodontine rodent-borne hantaviruses, including Sin Nombre virus,[3] New York virus,[4] Black Creek Canal virus,[5] Bayou virus,[6] and Andes virus,[7] have been identified in the Americas. New hantaviruses have also been discovered in Eurasia: Khabarovsk virus in the reed vole (*Microtus fortis*)[8]; Topografov virus in the Siberian lemming (*Lemmus sibiricus*)[9]; and Tula virus in the European common vole (*Microtus arvalis* and *M. rossiaemeridionalis*), initially in Central Russia and subsequently in the Slovak and Czech Republics, Austria, and Poland.[10–15]

Abundant clinical, serologic, and virologic data support the presence of Seoul, Dobrava–Belgrade, and Puumala viruses in the former Yugoslavia.[16–21] At the same time, atypical serum neutralizing antibody responses to prototype strains of Hantaan and Puumala

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viruses in some HFRS patients have long suggested the existence of other pathogenic hantaviruses in the Balkans.[16,19] Previously, the isolation of Belgrade virus from Yugoslavian patients with severe HFRS validated this conjecture.[22] The authors have now specifically targeted arvicolid rodents in the former Yugoslavia in search of Puumala-like viruses. This report indicates the presence of Tula virus in the European pine vole (*Pitymys subterraneus*; also known as *Microtus subterraneus*) in Serbia-Yugoslavia.

MATERIALS AND METHODS

Rodent trapping

From 1983 to 1989, rodent trappings were conducted at several sites within the former Yugoslavia: four sites in Bosnia, three sites each in Croatia and Serbia, and one site each in Montenegro and Slovenia. Rodent sera, diluted 1:32 in 0.1 M phosphate-buffered saline, were tested for IgG antibodies against Hantaan and Puumala viruses by the indirect immunofluorescent antibody (IFA) technique.[16]

Amplification of hantaviral RNA

Total cellular RNA, extracted from tissues of seropositive rodents using RNAzol (GIBCO/BRL, Gaithersburg, MD), was analyzed for hantaviral RNA by reverse transcript-polymerase chain reaction (rt-PCR). Briefly, following reverse transcription using the superscript II RNase H^- reverse transcriptase kit (GIBCO/BRL), cDNA was amplified by nested PCR, using previously described oligonucleotide primers,[23] as well as the following primers: for the S genomic segment, 5′-TGACAGA(A+G)TGGGGTTCTGAT-3′ +1208, 5′-GCCATCCC(A+T)GC(A+T)ACATAA AT-3′−1031, 5′-T(C+T)TCAGGCCAATCTTTAACAA-3′−745, 5′-TTGCACTACAGCTTGGTCATT-3′+1518, 5′-TACAGAGCAGCAGATTACCTGA-3′−1566, 5′-GCA AGTGCAAAGCCT(G+A)TAAT-3′−437; for the M genomic segment, 5′-CCT TGTCAGGTTGA (C+T)TTGAGTG-3′+2971, 5′-TGCTTATCCTTG-GCAGACAG-3′+1230, 5′-TACTG(G+A)CATGAT-GGTAATGT-3′−2784. Primers afforded amplification of the entire nucleocapsid protein-encoding S segment and a 948-nucleotide region of the G2 glycoprotein-encoding M segment.

Polymerase chain reaction products were cloned using the TA cloning system (Invitrogen Corp., San Diego, CA), and plasmid DNA was purified by the QIAprep-spin Plasmid Kit (QIAGEN Inc., Chatsworth, CA). DNA sequencing was performed in both directions from at least three clones of each PCR product, using the Dye Primer Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) on an automated sequencer (Model 373A).

Amplification of mitochondrial DNA

Total DNA was extracted from rodent lung tissues using the QIAamp Tissue Kit (QIAGEN). The cytochrome b and D-loop regions of mitochondrial DNA (mtDNA) were amplified by PCR, using previously described universal primers,[24,25] which permit amplification of 482-bp and 1020-bp products, respectively. Primers were used at a final concentration of 0.1 μM/100 μL reaction mixture (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTP) containing 5 μL of total DNA (25–100 μg/mL) and 2.5 units of AmpliTaq polymerase (Perkin Elmer Co., Norwalk, CT). Reaction mixtures were cycled 40 times: denaturation for 1 minute at 94°C, annealing for 1 minute at 55°C, and extension for 2 minutes at 72°C. Polymerase chain reaction products were cloned, and DNA was sequenced, as indicated above.

Genetic and phylogenetic analyses

Hantavirus sequences, amplified from a *Pitymys* rodent captured in the Cacak region of Serbia-Yugoslavia, were aligned and compared with previously published *Hantavirus* sequences. Alignment and comparison of the entire coding region of the nucleocapsid protein-encoding S segment and a 948-nucleotide region of the G2-encoding M segment were facilitated by using various programs of the GCG package (Genetics Computer Group Inc., University of Wisconsin, Madison, WI). For phylogenetic analysis, the neighbor-joining (N-J) method, unweighted pair-group method of assortment (UPGMA), Fitch-Margoliash method of PHYLIP (Phylogenic Inference Package, version 3.5c),[26] and maximum parsimony or PAUP (Phylogenetic Analysis Using Parsimony, version 3.1.1) were used.[27] The genetic distances were computed by the DNADIST program, using the Kimura two-parameter method and tree structures were inferred using the N-J, UPGMA, and Fitch-Margoliash methods with the global rearrangements option. Phylogenetic trees, based on hantaviral and mtDNA sequences, were evaluated statistically by calculating bootstrap probabilities for 1000 iterations.

RESULTS

Serologic survey

Of 1026 rodents, principally field mice (*Apodemus agrarius*, *Apodemus flavicollis*, and *Apodemus sylvaticus*), captured between 1983 and 1989 in multiple sites within the former Yugoslavia, IgG antibodies against Hantaan or Puumala viruses were detected in 12 of 31 (39%) *Pitymys subterraneus*, 1 of 5 (20%) *Pitymys* or *Microtus multiplex*, and 7 of 14 (50%) *Microtus arvalis*. Both *Pitymys subterraneus* and *Microtus arvalis* were captured in Stancici, near Cacak in Serbia, and in other

Table 1. Nucleotide and amino acid sequence homologies of the entire coding region of the S segment and of the partial G2 glycoprotein-encoding M segment between the *Pitymys*-borne Tula Virus (Strain Cacak) and other arvicolid rodent-borne hantaviruses

	S Segment		*M Segment*	
Virus Strain	*1290 nt (%)*	*430 aa (%)*	*948 nt (%)*	*316 aa (%)*
TUL/M5286	86.3	98.1	85.9	99.7
TUL/M5302	86.4	97.9	86.2	99.4
TUL/T53	85.3	97.7	80.7*	98.2*
TUL/T23	85.7	96.5	ND	ND
IV/MC-47	75.7	86.0	77.4*	88.4*
KBR/MF-43	ND	ND	73.4	83.2
PH/PH-1	76.1	81.6	72.1	82.0

*Comparison is based on 337 nucelotides (nt) and 112 amino acids (aa) in the G2 glycoprotein-encoding M segment.
TUL=Tula virus; IV=Isla Vista virus; KBR=Khabarovsk virus; PH=Prospect Hill virus; ND=no data available.

regions (e.g., Fojnica in Bosnia, and Slunji and Plitvice in Croatia).

Attempts to isolate hantavirus from lung tissues of four seropositive voles (three *Pitymys subterraneus* and one *Microtus arvalis*), by multiple passages in Vero E6 cells at two-week intervals, were unsuccessful.

Sequence analysis of *Pitymys*-borne hantavirus

The complete S segment and partial G2 glycoprotein-encoding M segment were amplified and sequenced from lung tissue from a *Pitymys subterraneus* captured in March 1987 following an epidemic of HFRS in Stancici (10 km N, 12 km E from Cacak, and 100 km S of Belgrade). The S genomic segment of this strain (designated Cacak) was 1834 nucleotides in length, with a predicted coding capacity of 430 amino acid (starting at nucleotide position 43) and a 502 nucleotide-long 3′-noncoding region. A second open reading frame was identified in the same position as in other arvicolid

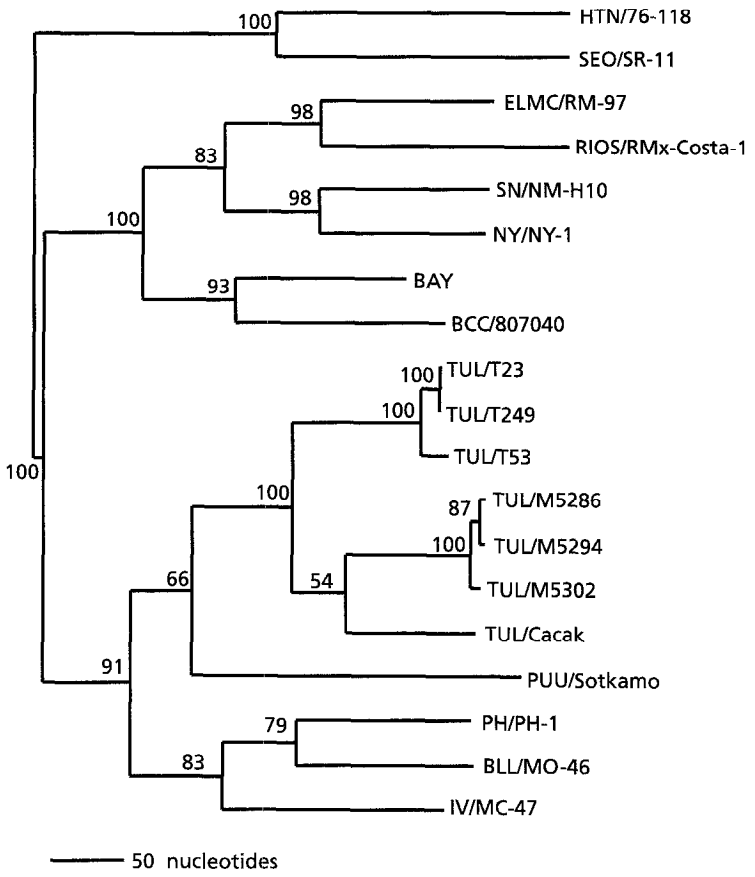

Figure 1. A majority-rule consensus phylogenetic tree, rooted at the midpoint, generated by the maximum parsimony method using PAUP, based on the entire coding region of the S segment of Tula (TUL) virus harbored by *Pitymys subterraneus* in Serbia-Yugoslavia (Cacak) (Genbank accession no. AF017659), and TUL virus strains amplified from *Microtus arvalis* and *Microtus rossiaemeridionalis* in central Russia (T23 and T53) and the Czech Republic (M5286, M5293 and M5302), showing phylogenetic clustering according to geographic origin. The phylogenetic positions of Hantaan (HTN), Seoul (SEO), El Moro Canyon (ELMC), Rio Segundo (RIOS), Sin Nombre (SN), New York (NY), Bayou (BAY), Black Creek Canal (BCC), Puumala (PUU), Prospect Hill (PH), Bloodland Lake (BLL), and Isla Vista (IV) viruses are also shown. Branch lengths are proportional to the number of nucleotide substitutions, whereas vertical distances are for clarity. The numbers at each node are bootstrap probabilities (expressed as percentages), as determined for 1000 iterations by PAUP version 3.1.1. Highly congruent topologies were derived from analysis of a 948-nucleotide region of the G2-encoding M genomic segment of TUL virus (Cacak strain) (Genbank accession no. AF017658).

rodent-borne hantaviruses, starting at nucleotide position 83 and encoding a 90-amino acid hypothetic nonstructural protein of unknown function, designated NSx.

The entire S-segment coding region of the *Pitymys*-borne hantavirus diverged from *Microtus* rodent-borne Tula virus strains by 13.6% to 14.7% at the nucleotide level (Table 1). However, the corresponding deduced amino acid sequences diverged by only 1.8% to 3.5%. Similarly, sequence analysis of a 948-nucleotide region of the G2 glycoprotein-encoding M segment similarly revealed that the Cacak strain differed by approximately 14% from other Tula virus strains, but the G2 glycoprotein was highly conserved.

In the hypervariable region of the nucleocapsid protein (amino acid residues 244 to 269), the Cacak strain differed by one or two amino acids from other Tula virus strains (Data not shown). The Moravia (M5286) and Malaky (Ma32) strains had a deletion at amino acid position 252, whereas the Cacak strain and Tula virus strains from Central Russia (e.g., T23) had an alanine at this position. Collectively, the data revealed that both point mutations and deletions accounted for genetic drift in Tula virus strains.

Phylogenetic analysis of *Pitymys*-borne hantavirus

A majority-rule consensus phylogenetic tree generated by PAUP, based on the entire S-segment coding region, showed that all Tula virus strains shared a common ancestry and indicated that the *Pitymys*-borne hantavirus from Serbia-Yugoslavia was more closely related to Tula virus strains from the Czech Republic than to those from Central Russia (Figure 1). Employing the maximum parsimony and N-J methods, as well as UPGMA, phylogenetic trees based on a 948-nucleotide region of the G2 glycoprotein-encoding M segment were highly congruent (data not shown) and supported

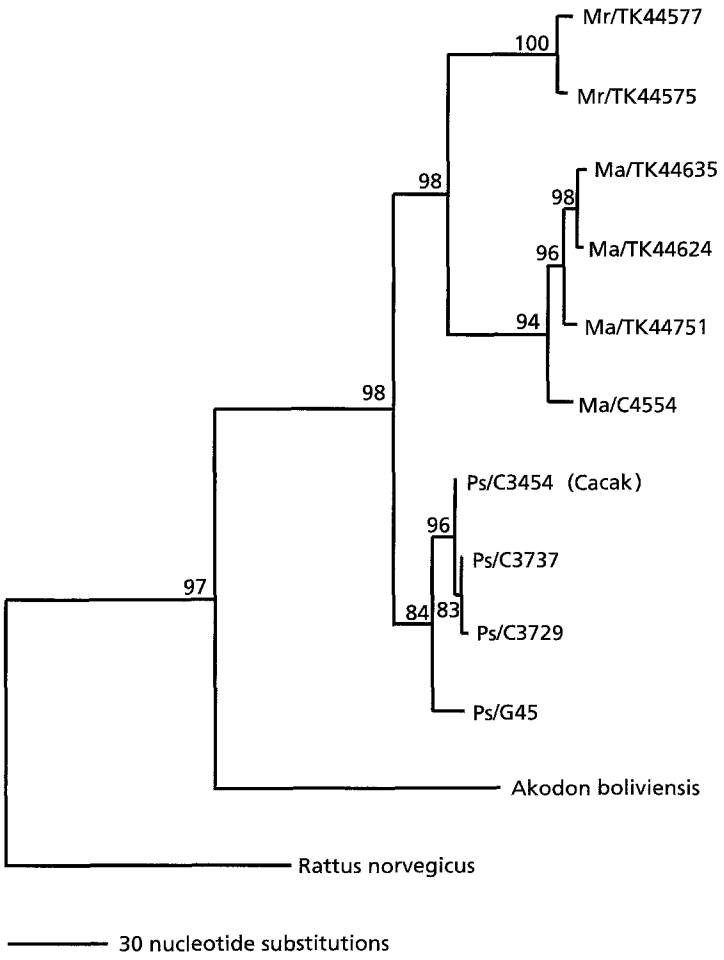

Figure 2. A phylogenetic tree based on a 426-bp region of cytochrome b-coding mtDNA, showing that *Pitymys subterraneus* (Ps) was genetically distinct from *Microtus arvalis* (Ma) and *Microtus rossiaemeridionalis* (Mr). Moreover, *Microtus* and *Pitymys* rodents clustered according to their geographic origin (Ps/C3454, Ps/C3737, and Ps/C3729 from Serbia-Yugoslavia; Ps/G45 from Poland; Mr/TK44577, Mr/TK44575, and Ma/TK44635, Ma/TK44624, and Ma/TK44751 from Chernobyl, Ukraine; and Ma/C4554 from Serbia-Yugoslavia). The phylogenetic postions of *Akodon boliviensis* and *Rattus norvegicus* are also shown. Tula (TUL) *Hantavirus* sequences, reported here, were amplified from Ps/C3454. Mitochondrial DNA sequences have been deposited in Genbank: Ps/C3454 (AF017660); Ps/C3729 (AF017661); Ps/C3737 (AF017662); Ps/G45 (AF017657); Ma/C4554 (AF017755).

geographic-specific clustering of Tula virus. All topologies were extremely robust, as supported by bootstrap analysis of 1000 iterations.

Rodent mtDNA sequence analysis

The vole captured near Cacak, from which Tula hantavirus sequences were amplified, was identified as *Pitymys subterraneus* by morphologic criteria. This was verified by mtDNA sequence analysis. Phylogenetic analysis of a 426-bp cytochrome b region of mtDNA showed that *Pitymys subterraneus* was genetically distinct from *Microtus arvalis* and *Microtus rossiaemeridionalis*, and that *Microtus* and *Pitymys* rodents tended to cluster according to their geographic origin (Figure 2). Moreover, *Pitymys* voles from Serbia-Yugoslavia and Poland were distinct, as were *Microtus* rodents from Russia and Serbia-Yugoslavia.

DISCUSSION

Since 1952, when four cases of nephropathia epidemica were reported in the Balkans,[28] more than 3000 cases of HFRS have been recorded in the former Yugoslavia, with mortality rates exceeding 10% in some regions.[19] In 1989, the authors reported the isolation of Hantaan virus and Puumala virus from the yellow-necked field mouse (*Apodemus flavicollis*) and the bank vole (*Clethrionomys glareolus*), respectively, captured in HFRS-endemic regions in Yugoslavia.[16] The subsequent independent isolation of Belgrade virus,[22] from blood and urine specimens of Yugoslavian patients with severe HFRS, and of Dobrava virus,[29] from lung tissue of *Apodemus flavicollis* captured in Slovenia, was consistent with serologic data that suggested the existence of other antigenically distinct pathogenic hantaviruses in the Balkans. Dobrava–Belgrade viral RNA has since been detected in blood samples of HFRS patients in Albania and Greece,[30,31] indicating that this disease-causing *Apodemus*-borne hantavirus is endemic in the Balkans.

Seoul virus infection has also been demonstrated in the former Yugoslavia. In 1992, a Canadian soldier was infected with Seoul virus in Sarajevo, Bosnia, and subsequently, Seoul virus infection was confirmed in a British soldier deployed to this region.[20] During the recent conflict in Bosnia and Herzegovina, more than 300 HFRS cases have been reported in the Tuzal region.[32,33] Most of these case-patients have exhibited typical serologic responses to Hantaan and Puumala viruses. Thus, Dobrava-Belgrade, Seoul, and Puumala viruses have previously been identified in the former Yugoslavia.

This report demonstrates that Tula virus, harbored by *Pitymys subterraneus*, is the fourth genetically distinct hantavirus circulating in the former Yugoslavia. Originally identified in *Microtus arvalis* and *Microtus rossiaemeridionalis* captured in the Tula region of Central Russia,[10] Tula virus has since been identified in *Microtus arvalis* captured in the Slovak and Czech Republics, Austria and Poland.[11–15] The *Pitymys*-borne Tula virus strain, reported here, shared a common ancestry with *Microtus*-borne Tula virus strains, but was more closely related to strains from the Czech Republic than those from Russia. The degree of genetic variability in the S genomic segment between Tula virus strains from Serbia-Yugoslavia, Central Russia, and the Czech Republic was similar to that for Puumala virus strains from Finland and Russia.[34,35] Moreover, as in *Clethrionomys*-borne strains of Puumala virus, sequence and phylogenetic analyses indicated geographic-specific grouping of *Microtus*- and *Pitymys*-borne Tula virus strains.

Since *Pitymys subterraneus* and *Microtus arvalis* are morphologically similar, they are occasionally difficult to differentiate, particularly in geographic regions where they are sympatric and synchronistic. One of the rodents (Ma/C4554) captured in the Cacak region was originally classified as *Pitymys subterraneus* by morphologic criteria, but proved to be *Microtus arvalis* by mtDNA sequence analysis. However, as determined by morphologic features and verified by mtDNA sequence analysis, the rodent from which Tula hantaviral RNA was amplified was identified as *Pitymys subterraneus*. Thus, the European pine vole may also serve as a rodent reservoir of Tula virus in Serbia-Yugoslavia. To what extent this represents virus spillover from *Microtus arvalis* warrants further investigation.

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